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ARTIFICIAL MATRIX ATTACHMENT REGION FOR INCREASING EXPRESSION OF GENES INTRODUCED IN PLANT CELLS

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Synthetic DNA molecule is useful as matrix attachment region to increase expression of genes introduced in transformed plants.

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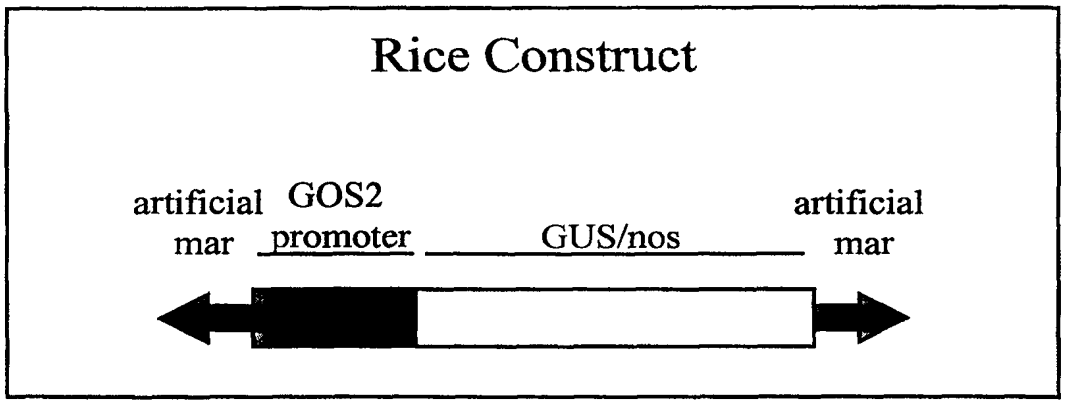

(57) Abstract

Synthetic DNA molecule is useful as matrix attachment region to increase expression of genes introduced in transformed plants.

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ARTIFICIAL MATRIX ATTACHMENT REGION FOR INCREASING EXPRESSION OF GENES INTRODUCED IN PLANT CELLS

The present invention relates to plant molecular biology, and in particular to technology for enhancing the expression of genes introduced in transformed plant cells.

Through the use of recombinant DNA technology and genetic engineering, it has become possible to introduce desired DNA sequences into plant cells to allow for the expression of proteins of interest. Plants with genetically engineered traits, such as, for example, insect resistance, disease resistance, drought resistance, herbicide resistance, or metabolic alterations that increase or modify production of useful plant products, offer great promise of improving agriculture.

Obtaining desired levels of expression of DNA introduced into plant cells remains a challenge. One problem, referred to as "position effect" variation, is the variation in expression of the same gene in independent transformants. The use of naturally occurring DNA sequences called matrix attachment regions or scaffold attachment regions to combat this problem was proposed in U.S. Patent 5,773,689 and in WO 94/24293.

The present invention provides a novel synthetic DNA molecule comprising bp 11 to 309 of SEQ ID NO: 1 that is useful as a matrix attachment region to increase expression of genes introduced in transformed plants.

In another of its aspects, the invention provides a DNA construct comprising, in the 5' to 3' direction: a transcription initiation region functional in plant cells, a structural gene operatively associated with the transcription initiation region, a 3' untranslated region, and a matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 positioned either 5' to said transcription initiation region or 3' to said structural

gene. In a preferred embodiment, a first matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 is positioned 5' to said transcription initiation region and a second matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 is positioned 3' to said 3' untranslated region.

In a particularly preferred embodiment, the matrix attachment region of the invention comprises two or more tandem copies of bp 11 to 309 of SEQ ID NO:1.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram showing the strategy for assembling the artificial MAR of SEQ ID NO:1.

FIG. 2 is a schematic representation of the rice transformation construct ArGOS2Af, which contains the MAR dimer.

FIG. 3 is a graph comparing relative GUS activity for multiple rice transformation events using non-MAR containing construct GOS2 and construct ArGOS2Af, which contains an artificial MAR dimer.

FIG. 4 is a graph showing the effect of the artificial MAR on ranges of expression of the GUS reporter gene in transgenic rice plants.

FIG. 5 is a schematic representation of the *Arabidopsis* transformation constructs ArAct2Af and aaaaAfAct2Af. ArAct2Af contains copies of the MAR dimer in opposite orientations flanking the reporter gene. AfAct2Af contains copies of the MAR dimer in the same orientation flanking the reporter gene.

FIG. 6 is a graph comparing relative GUS activity for multiple *Arabidopsis* transformation events using non-MAR containing construct Act2 and constructs ArAct2Af and AfAct2Af, which contain an artificial MAR dimer.

FIG 7 is a graph showing the effect of the artificial MAR on the range of expression of the GUS reporter gene in transgenic *Arabidopsis* plants.

DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 describes the artificial MAR of the invention.

SEQ ID NOS:2 to 4 describe ARBP sites.

SEQ ID NO:5 describes an ATF site.

SEQ ID NO:6 describes a BEAF-32 site.

SEQ ID NOS:7 to 9 describe topoisomerase II sites.

SEQ ID NO:10 describes an unwinding sequence.

SEQ ID NOS:11 to 17 describe SATB I sites.

SEQ ID NO:18 describes exemplary bending DNA.

SEQ ID NO:19 describes an exemplary A/T tract.

SEQ ID NO:20 describes synthetic MAR-A.

SEQ ID NO:21 describes synthetic MAR-B.

SEQ ID NO:22 describes synthetic MAR-C.

SEQ ID NO:23 describes synthetic MAR-D.

SEQ ID NO:24 describes synthetic MAR-E.

SEQ ID NO:25 describes synthetic MAR-F.

SEQ ID NO:26 describes the 3' MAR dimer in pArGOS2Af-hpt and ArAct2Af-bin

SEQ ID NO:27 describes rice transformation vector pGOS2-hpt.

SEQ ID NO:28 describes rice transformation vector pArGOS2Af-hpt.

SEQ ID NO:29 describes the 5' MAR dimer in pArGOS2Af-hpt and ArAct2Af-bin.

SEQ ID NO:30 describes dicot transformation vector pAct2-bin.

SEQ ID NO:31 describes dicot transformation vector pArAct2Af-bin.

SEQ ID NO:32 describes dicot transformation vector pAfAct2Af-bin.

SEQ ID NO:33 describes the 3' MAR dimer in pAfAct2Af-bin.

DETAILED DESCRIPTION OF THE INVENTION

Eukaryotic nuclei are highly organized structures in which the entire genetic information has to be accessible in an orderly manner for replication, transcription and

other cellular events (Lewin, 1994; Dillon and Grosveld, 1994; Jackson, 1995; Wolffe, 1994). Genes are typically organized in chromatin loops of various sizes that are attached to the proteinaceous nuclear matrix at locations known as matrix attachment regions (MARs). MARs are often located in non-transcribed regions of genes and are thought to form the physical boundaries of individual DNA loops. In several cases, MARs were shown to reduce position effect in transgenic organisms. The chicken lysozyme MAR was shown to increase expression, reduce variance and make expression of an adjacent gene copy number dependent in stably transfected cells (Stief *et al.*, 1989) in transgenic mice (Bonifer *et al.*, 1990, McKnight *et al.*, 1992) and in transgenic tobacco plants (Mlynárová *et al.*, 1994; Mlynárová *et al.*, 1995).

However, not all MARs have these effects on gene expression. Two minimal *Drosophila* MARs (one located between the histone H1 and H3 genes, and the other near the heat shock HSP70 genes) stimulated expression more than 10-fold in stably transformed cells, but the presence of these MARs did not reduce position effect (Poljak *et al.*, 1994). MARs from the apolipoprotein domain increased expression and reduced position effect in low-copy transformants, but expression in multicopy transformants was strongly repressed. (Kalos and Fournier, 1995). When a *Drosophila ftz* MAR was placed in a different chromosomal location, it did not reorganize chromatin structure and the chromatin fragment containing the MAR could be easily eluted from the nucleus, indicating that introduced MARs do not necessarily form chromatin domains (Eggert and Jack, 1991). In contrast, MARs flanking the immunoglobulin m heavy chain locus enhancer were required for high levels of expression and the formation of an extended DNase I sensitive domain in transgenic B lymphocytes, but not in stably transfected tissue culture cells (Forrester *et al.*, 1994).

Results using MARs in transgenic plants have been similarly complex (Spiker and Thompson, 1996). A yeast MAR increased expression levels in stably transformed tobacco callus lines, but no correlation between copy number and expression level could be found (Allen *et al.*, 1993). In contrast, the MAR element from the soybean heat shock gene *Gmhsp17.6-L* was shown to be capable of increasing expression levels but had little effect on variability (Schöffl *et al.*, 1993). A soybean MAR flanking a reporter gene construct reduced variability of expression when compared to a construct lacking MARs, but also reduced expression levels when present 5' and 3' of a reporter gene construct in transgenic tobacco callus (Breyne *et al.*, 1992). It is possible that individual MARs can have different functional and structural properties in addition to their matrix binding ability (Breyne *et al.*, 1994).

MARs are usually 300 to 2000 base pairs in length, are rich in adenosine and thymine residues and often contain certain conserved sequence elements and structural features. Most MARs described in the literature are not obtained from plants, but it has been well documented that MARs from other organisms bind plant scaffolds and vice versa (Dietz *et al.*, 1994; Breyne *et al.*, 1992).

Table 1 describes sequence elements present in MARs described in the literature, including the following plant MARs: soybean heat shock protein gene MAR, (Schöffl *et al.*, 1993); a petunia MAR (Dietz *et al.*, 1994); the pea plastocyanin gene MAR (Slatter *et al.*, 1991); the maize Adh1 gene 5′ and 3′ MARs (Avramova and Bennetzen, 1993; Avramova *et al.*, 1995); the b-phaseolin gene 5′ and 3′ MARs (van der Geest *et al.*, 1994.

Table 1

element	characteristics	sequence	SEQ ID NO:	reported in plant MARs
ARBP	ARBP (attachment-region binding protein) from chicken which binds MARs from *Drosophila*, mouse, chicken and human genes in a cooperative manner (von Kries *et al.*, 1991; Buhrmester *et al.*, 1995)	ATTTCASTTGTAAAA TGCAGGTGTCCTT TGGGGGTGTAAAA	2 3 4	no yes yes
ATF	ATF sequences bind transcription factors of the ATF family, two of which were shown to be integral components of the nuclear matrix (Stein *et al.*, 1991)	TGACGTCCATG	5	no
BEAF-32	Beaf-32 (boundary element-associated factor of 32 kDa molecular mass) binds to the locus boundary element scs' from *Drosophila*, which can insulate reporter genes from position effect variegation (Zhao *et al.*, 1995)	CGATA	6	yes
topo-isomerase II	Topoisomerase II is a major component of the nuclear matrix and topoisomerase II binding sites have been found in most MARs to date (Sander and Hsieh, 1985; Boulikas, 1995)	CNNGYNGKTNYNY ASMATGCGYWYATCRT GTNWAKATTNATNNR	7 8 9	yes no yes
unwinding sequence	Unwinding sequences such as AATATATTT cause DNA unwinding *in vivo*, which is important for MAR function (Bode *et al.*, 1992)	AATATATTT	10	yes
SATB1	SATB1 is a protein isolated from human thymus tissue that selectively binds MAR sequences consisting of A's, T's and C's in one strand (Dickinson *et al.*, 1992; Nagagomi *et al.*, 1994)	TTCTAATATAT ATAATCTTC TTATTATTTA TATAAAAA AAGATTATATA TTTTAATGAGATAATAA TATAATCTTC	11 12 13 14 15 16 17	no no yes yes no no no
bending DNA	Curved DNA regions are often found in or near MARs (Bode *et al.*, 1995)	AAANNNNNNNAAA	18	yes
stem-loop	Stem loops create small single-stranded regions which are important for MAR function (Boulikas and Kong, 1993)			yes
oligo A/T tracts	These can create bent DNA that may attract protein complexes involved in topoisomerization,	AAAAAAA	19	yes

element	characteristics	sequence	SEQ ID NO:	reported in plant MARs
	recombination, transcription or replication (Travers, 1990)			

K: G or T, M: A or C, N: A, C, G or T, R: A or G, S: C or G, W: A or T, Y: C or T.

The present invention utilizes a subset of the features described in Table 1 in a novel artificial MAR. The sequence of the 327 bp artificial MAR is given in SEQ ID NO:1. The artificial MAR was designed as a sequence flanked by *Bgl*II and *Bam*HI restriction sites, which are included in SEQ ID NO:1, but which are not critical to the function of the MAR. The functional portion of the MAR comprises bp 11 to 309 of SEQ ID NO:1.

The following features are found in SEQ ID NO:1:

Feature	location (bp)
*Bgl*II	5-10
BEAF-32	11-15
SATBI	24-34
unwinding	28-36
topoisomerase II	44-59
ATF site	60-69
A/T tract	70-85
BEAF-32	86-90
stem-loop	93-101/117-124
unwinding	105-113
topoisomerase II	125-139
SATBI	149-159
unwinding	153-161
BEAF-32	164-168
SATBI	185-195
stem-loop	208-216/231-239
unwinding	219-227
A/T tract	241-253
topoisomerase II	268-283
curved (bending) DNA	284-294
ARBP site	295-309
BamHI	318-323

The 3' UTR, or 3' untranslated region, that is employed in constructs of the invention is one that confers efficient processing of the mRNA, maintains stability of the message and directs the addition of adenosine ribonucleotides to the 3' end of the transcribed mRNA sequence. The 3' UTR may be native with the promoter region, native with the structural gene, or may be derived from another source. Suitable 3' UTRs include, but are not limited to: the *per5* 3' UTR, and the 3' UTR of the nopaline synthase (*nos*) gene.

Example 1

Synthesis of artificial MAR

To construct the artificial MAR, six individual oligonucleotides were synthesized and assembled by PCR. The sequences for the six oligonucleotides, referred to hereinafter as MAR-A, MAR-B, MAR-C, MAR-D, MAR-E, and MAR-F, are given in the Sequence Listing as SEQ ID NOS: 20 through 25, respectively. A 15 bp overlap between adjacent oligonucleotides allowed assembly of the MAR by PCR, using the strategy shown in Figure 1.

The GeneAmp™ PCR Reagent Kit with AmpliTaq DNA Polymerase (Perkin Elmer, Norwalk, CT) was used for the DNA amplification. Twenty cycles of PCR (denaturation: 30 sec at 94°C; annealing: 60 sec at 52°C and extension: 60 sec at 70°C) with primers MAR-C and MAR-D were followed by 20 cycles of PCR with primers MAR-B and MAR-E, using the product from the first reaction as template for the second reaction. The 231 bp product of this reaction was purified from a low melting point agarose gel and used as a template for 20 cycles of PCR with primers MAR-A and MAR-F. The 327 bp product from this reaction was subcloned into pCR2.1 using the TA Cloning™ Kit (Invitrogen, San Diego, CA) and the sequence was verified by sequencing.

A dimer consisting of two tandem copies of the *Bgl*II/*Bam*HI fragment was constructed in the *Bam*HI site of pBluescript™ SK- (Stratagene, La Jolla, CA). The sequence of the dimer is bp 5-630 of SEQ ID NO:26.

EXAMPLE 2

Binding of artificial MAR to nuclear scaffolds

A. Controls

Two DNA fragments of similar size and nucleotide composition as the artificial MAR were amplified from plant DNA to serve as controls in the binding assay. These fragments were a 657 bp fragment from the 3' end of a maize gene Gpa1 (glyceraldehyde-3-phosphate dehydrogenase subunit A, GenBank accession number X15408, bases 4516 to 5173, Quigley *et al.*, 1989), and a 488 bp fragment from the 5' flanking region of a 19 kD alpha zein gene (GenBank accession number X05911, bases 339 to 827, Kriz *et al.*, 1987). Table 2 compares the features present in the artificial MAR and control fragments.

Table 2

element	Artificial MAR dimer	Gpa1 control	zein control
ARBP sites	2	0	0

element	Artificial MAR dimer	Gpa1 control	zein control
ATF sites	2	0	0
BEAF-32 sites	6	1	1
topoisomerase II sites	6	0	0
unwinding sequence sites	8	0	0
SATB1 sites	6	0	0
bending DNA sites	yes	yes	yes
stem-loop sites	4	1	0
oligo A/T tracts	2	0	1
fragment size in binding assay	632	657	488
% A + T	71	63	66
strength of binding to nuclear scaffolds	+++++	-	-

B. Preparation of nuclei from maize leaves

Nuclei for use in isolating nuclear scaffolds were prepared from young maize leaves by adaptation of a published protocol (Hall *et al.*, 1991). Nuclei were counted and checked for integrity by microscopic examination of DAPI stained aliquots. Only high quality nuclei were used to prepare nuclear scaffolds by lithium diiodosalicylate extraction.

For nuclei purification, young maize leaves from V4 stage plants (fourth or fifth leaf) were harvested with a razor blade, washed and dried. After removing the midrib, leaves were frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The powdered leaf samples were transferred to a glass beaker, and 5 ml NIB1+PI (0.5 M hexylene glycol, 20 mM piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES), pH 6.5, 20 mM KCl, 7 mM 2-mercaptoethanol, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.4 % Triton X-100™ [Rohm & Haas Company, Philadelphia, PA], 0.05 mM spermine, 0.125 mM spermidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin) was added per gram of leaf tissue. The leaf extract was filtered sequentially through 1900, 520, 125, 85 and 40 mm filters at 4°C and filters were rinsed with 1 ml NIB1+PI per gram leaf to collect any nuclei that were trapped in the debris. Fifteen ml crude nuclear extract was loaded onto Percoll™ (Pharmacia Biotech, Piscataway, NJ) gradients consisting of 7 ml 40% Percoll in NIB1 (0.5 M hexylene glycol, 20 mM PIPES, pH 6.5,

20 mM KCl, 7 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.4 % Triton™ X-100, 0.05 mM spermine, 0.125 mM spermidine) and 5 ml 70% Percoll in NIB1. After centrifugation for 15 min at 500x*g* at 4°C the 40%/70% interface was collected with a sterile pasteur pipette and added to 2 volumes NIB2 (0.5 M hexylene glycol, 20 mM PIPES, pH 6.5, 20 mM KCl, 7 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine), taking care to avoid the pellet and other debris. Nuclei were concentrated by centrifugation at 600x*g* for 10 min at 4°C.

The nuclear pellet was resuspended in 20 ml NIB2 and centrifuged as before. This step was repeated one more time to wash away traces of Percoll. Nuclei were counted using a hemacytometer and resuspended in NIB2+PI/50% glycerol (0.5 M hexylene glycol, 20 mM PIPES, pH 6.5, 20 mM KCl, 7 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, 50% glycerol, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) at 20 million nuclei/ml. Nuclei were stored at -80°C until used for scaffold preparation.

C. Preparation of nuclear scaffolds

Frozen nuclei were thawed and washed with 10 ml of NIB3+PI (0.5 M hexylene glycol, 20 mM PIPES, pH 6.5, 20 mM KCl, 7 mM 2-mercaptoethanol, 0.05 mM spermine, 0.125 mM spermidine, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) per 20 million nuclei. Nuclei were collected by centrifugation at 600x*g* for 10 minutes, resuspended in 200 ml NIB3+PI in the presence of 1 mM $CuSO_4$, and incubated for 10 min at 42°C to stabilize the nuclei.

Histones were extracted by incubation in 10 ml HIB+PI (20 mM HEPES, pH 7.4, 100 mM lithium acetate, 10 mM LIS (lithium diiodosalicylate), 0.1 % digitonin, 2 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) for 15 minutes at room temperature. The resulting nuclear halos were transferred to a centrifuge tube and pelleted at 4000x*g* for 10 minutes. Halos were washed twice with 10 ml HWB (20 mM Tris, pH 8, 70

mM NaCl, 20 mM KCl, 7 mM 2-mercaptoethanol, 0.1 % digitonin, 0.05 mM spermine, 0.125 mM spermidine) and once with D/BB+PI (HWB + 10 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin) to remove LIS. If halos did not pellet well, subsequent centrifugation steps were done at 6000x*g* using a slow brake setting. The quality of the halos was verified by SDS-PAGE gel to ensure that more than 95% of the histones were removed in the extraction procedure

Washed nuclear halos were resuspended in 400 μl D/BB+PI and 200 units of restriction enzymes (100 u each of *Eco*RI and *Hind*III) were added, and incubated at 37°C for 2-3 hours on a rocking platform to keep the halos from settling. The restriction enzymes removed more than 70% of the nuclear DNA, producing nuclear scaffolds. Scaffolds were pelleted at 300x*g* and washed with HWB+PI (HWB + 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Nuclear scaffolds were resuspended in 400 μl HWB+PI and separated into 100 μl aliquots (containing 5 million nuclear equivalents).

D. Binding of artificial MAR to nuclear scaffolds

100 μl aliquots of scaffolds in HWB+PI were incubated with probe and *E. coli* competitor DNA at 37°C for 2-3 hours in siliconized microfuge tubes on a rocking platform shaker. After incubation, the supernatant fraction (containing unbound DNA fragments) and pellet fraction (containing scaffolds and bound DNA fragments) were separated via centrifugation in a horizontal microfuge at 3000x*g* for 5 min. The pellet was washed once with 200 μl HWB to remove proteinase inhibitors, resuspended in 100 μl lysis buffer (10 mM Tris, pH 8, 10 mM EDTA, 0.5 % SDS, 0.5 mg/ml Proteinase K) and incubated overnight at room temperature.

Equal fractions of the pellet and supernatant were separated on a 0.9% agarose gel, which was subsequently fixed, by soaking in 7% TCA for 20 min, dried and exposed to X-ray

film at room temperature and/or storage phosphor screens for the PhosphoImager™ SI (Molecular Dynamics, Sunnyvale, CA).

Plasmids containing the artificial MAR monomer or dimer or the control Gpa1 or zein sequences were digested with restriction enzymes that generate 5′ overhang ends. The Klenow subunit of DNA polymerase I was used to fill the overhang with [a-^{32}P]dCTP (Amersham Life Science, Arlington Heights, IL). The end-labeled DNA fragments were used as probes in the binding assay, i.e. the fragments were incubated with purified maize nuclear scaffolds in the presence of unlabeled *E.coli* competitor DNA and the relative binding of the inserts was determined. Relative amounts of nuclei, probe and unlabeled *E. coli* competitor DNA used in the binding assay were optimized to obtain maximal discrimination between strongly and weakly binding MARs. The optimal relative amounts were 2, 5 or 10 μg of unlabeled *E. coli* competitor DNA, 5 million nuclear equivalents of nuclear scaffolds, and 1 fmole of digested and labeled plasmid per assay.

The artificial MAR dimer bound very strongly to the nuclear scaffold preparation, even in the presence of high levels of competitor DNA. The monomer MAR also bound to nuclear scaffold preparations, albeit at a lower affinity. Neither control sequence was retained in the pellet fraction, even though they were similar to the artificial MARs in size and relative AT content. This suggests that the elements included in the artificial MAR facilitate binding.

EXAMPLE 3

EVALUATION OF THE ARTIFICIAL MAR IN RICE

A. Rice Transformation Vectors

pGOS2-hpt (SEQ ID NO:27) is a rice transformation vector containing a hygromycin selectable marker driven by the 35S promoter and a GOS2/GUS/nos cassette (GOS2 transcription initiation region/GUS structural gene/nos 3' untranslated region). The GOS2 transcription inititation region in this construct is comprised of 1010 bp of promoter and 170 bp of

untranslated 5' leader interrupted by a 1100 bp intron (de Pater *et al.*, 1992).

pArGOS2Af-hpt (SEQ ID NO:28) is a rice transformation vector identical to pGOS2-hpt except that it has the MAR dimer of SEQ ID NO:29 positioned 5' to the GOS2 transcription initiation region and the MAR dimer of SEQ ID NO:26 positioned 3' to the nos 3' UTR.

A schematic representation of the ArGOS2Af construct is shown in FIG 2.

B. Transformation of Rice

For initiation of embryogenic callus, mature seeds of a *Japonica* cultivar, Taipei 309, were dehusked and surface-sterilized in 70% ethanol for 5-7 min. followed by soaking 30-45 min in 25% commercial bleach (2.6% sodium hypochlorite) with 0.02% Tween™ 20 (ICI Americas, Inc.) under vacuum. The seeds were then rinsed 5 times in sterile distilled water and placed on filter paper before transferring to induction media (NB). The NB medium consisted of N6 macro elements (Chu, 1978), B5 micro elements and vitamins (Gamborg *et al.*, 1968), 300 mg/l casein hydrolysate, 500 mg/l L-proline, 500 mg/l L-glutamine, 30 g/l sucrose, 2 mg/l 2,4-dichloro-phenoxyacetic acid (2,4-D), and 2.5 g/l Gelrite™ (Merck & Co., Rawhay, NJ) with the pH adjusted to 5.8. The mature seed cultured on induction media were incubated in the dark at 28° C for three weeks. Primary callus induced from the scutellar region of mature embryo was transferred to fresh NB medium for further maintenance and thereafter maintained on a two week subculture period.

To prepare DNA for blasting, about 140 µg of plasmid DNA (pGOS2-hpt or pArGOSAf-hpt) was precipitated onto 60 mg of gold particles. The plasmid DNA was precipitated onto 1.5-3.0 micron (Aldrich Chemical Co., Milwaukee, WI) or 1.0 micron gold particles (Bio-Rad Laboratories, Hercules, CA). The precipitation mixture included 60 mg of pre-washed gold particles, 300 µl of water/DNA (140 µg), 74 µl of 2.5 M $CaCl_2$, and 30 µl of 0.1 M spermidine. After adding the components in

the above order, the mixture was vortexed immediately, and allowed to settle for 2-3 min. The supernatant was pipetted off and discarded. The DNA-coated gold particles were resuspended in 1 ml of 100% ethanol and diluted to 17.5 mg DNA/7.5 mg gold per ml of ethanol for use in blasting experiments.

For helium blasting, actively growing embryogenic callus cultures, 2-4 mm in size, were subjected to a high osmoticum treatment by placing callus on NB medium with 0.2 M mannitol and 0.2 M sorbitol (Vain *et al.*, 1993) for 4 hr before helium blasting. Following osmoticum treatment, callus cultures were transferred to blasting medium (NB+2% agar) and covered with a stainless steel screen (230 micron). Helium blasting involved accelerating the suspended DNA-coated gold particles towards and into the prepared tissue targets. The device used was an earlier prototype to the one described in US Patent No. 5,141,131, which is incorporated herein by reference, although both function in a similar manner. The callus cultures were blasted at different helium pressures (1750-2,250 psi) one to three times per target. After blasting, callus was transferred back to the high osmotic media overnight before placing on selection medium, which consisted of NB medium with 30 mg/l hygromycin. After 2 weeks, the cultures were transferred to fresh selection medium with higher concentrations of selection agent, i.e., NB+50 mg/l hygromycin (Li *et al.*, 1993).

Compact, white-yellow, embryogenic callus cultures, recovered on NB+50 mg/l hygromycin, were regenerated by transferring to pre-regeneration (PR) medium+50 mg/l hygromycin. PR medium consisted of NB medium with 2 mg/l benzyl aminopurine (BAP), 1 mg/l naphthalene acetic acid (NAA), and 5 mg/l abscisic acid (ABA). After 2 weeks of culture in the dark, they were transferred to regeneration (RN) medium. The composition of RN medium is NB medium with 3 mg/l BAP, and 0.5 mg/l NAA. The cultures on RN medium were incubated for 2 weeks at 28° C under high fluorescent light

(325-ft-candles). The plantlets with 2 cm shoots were transferred to 1/2 MS medium (Murashige and Skoog, 1962) with 1/2 B5 vitamins, 10 g/l sucrose, 0.05 mg/l NAA, 50 mg/l hygromycin and 2.5 g/l Gelrite™ adjusted to pH 5.8 in GA7 vessels (Magenta Corp., Chicago, IL). When plantlets were established with well-developed root systems, they were transferred to soil [1 part Metro-Mix 360 (Scotts-Sierra Horticultural Products Co., Marysville, OH) and 1 part top soil) and raised in a growth chamber (29/24°C day/night cycle, 50-60% humidity, 12 h photoperiod) until they reached a height of 60 cm, at which point 2 leaves were harvested for quantitative GUS analysis, and the plants were transferred to the greenhouse to grow to maturity.

C. Southern analyses

Southern analysis was used to identify primary regenerate (R_0) rice lines lines that contained intact copies of the specific gene construct.

A DNA probe specific for the coding region of the β-glucuronidase (GUS) gene construct was gel purified with the Qiaex II DNA purification kit (Qiagen Inc., Chatsworth, CA). Radiolabeled probe was prepared using the Ready-To-Go™ DNA labeling beads (Pharmacia LKB, Piscataway, NJ) with 50 microcuries of [α^{32}P]dCTP (Amersham Life Science, Arlington Heights, IL).

Leaf material from R_0 rice plants was harvested from two representatives from each line. Genomic DNA from the R_0 plants was prepared from lyophilized tissue as described by Saghai-Maroof *et al.* (1984).

Four micrograms of rice DNA was digested with restriction enzyme to release the intact gene construct using conditions suggested by the manufacturer (Bethesda Research Laboratory, Gaithersburg, MD) and separated by agarose gel electrophoresis. The DNA was blotted onto nylon membranes as described by Southern (1975, 1989). Radiolabeled probe DNA was hybridized to the genomic DNA on the blots using 50 ml of

minimal hybridization buffer (10% polyethylene glycol, 7% sodium dodecyl sulfate, 0.6x SSC, 10 mM sodium phosphate, 5 mM EDTA and 100 mg/ml denatured salmon sperm DNA) heated to 60°C and mixed with the denatured radiolabeled probed prior to being added to the blots for overnight hybridization at 60°C. The blots were washed at 60°C in 0.25X SSC and 0.2% SDS for 45 minutes, blotted dry and exposed to XAR-5 film with two intensifying screens overnight.

Southern analysis was conducted on seventy ArGOS2Af R_0 rice lines. The DNA from the R_0 plants was digested with the restriction enzyme *Xba*I which, if the intact gene construct is present, should result in a 5.7 kb hybridization product when radiolabeled with a probe specific for the GUS coding region. The 5.7 kb fragment should consist of the artificial MAR in the reverse orientation, the GOS2 promoter, the GUS coding region, the nos 3′ UTR and the artificial MAR in the forward orientation. The expected 5.7 kb hybridization product was detected in twenty-five of the seventy rice lines. All of the twenty-five lines had multiple hybridization products and two of the lines had identical complex hybridization patterns indicating that they are probably from the same transformation event.

The non-Mar control lines, GOS2, were also analyzed by Southern analysis. The DNA from forty-eight GOS2 R_0 lines was digested with the restriction enzymes *Eco*RI and *Xba*I which, if the intact gene is present, should result in a 4.4 kb hybridization product when radiolabeled with a probe specific for the GUS coding region. The 4.4 kb fragment would include 1.6 kb of the GOS2 promoter, the GUS coding region, the nos 3′ UTR and the 35T promoter (the promoter used to drive the selectable marker gene). The expected 4.4 kb hybridization product was detected in twenty-eight of the forty-eight GOS2 lines. Two of the lines had identical hybridization patterns and must have resulted from the same transformation event. Two of the lines contained genetic chimeras.

D. GUS analysis

Analysis of rice was performed on young leaves of primary transformants, after plants had been grown 6-8 weeks in an environmentally controlled growth chamber and had reached a height of about 60 cm. Two independently regenerated rice plants were analyzed per transformation event. Individual transformants were analyzed by Southern blots to verify the presence of an intact copy of the transgene and determine whether each event displayed unique hybridization patterns, indicating independent transformation events. Plants lacking a complete copy of the transgene, chimeric events, or duplicated integration events were not included in the analysis.

Results of the analysis are reported in FIGS 4 and 5. In FIG 4, error bars represent the standard deviation between the plants for each transformation event. Two samples were independently processed for each plant. In general, the level of expression of GUS in independent rice plants from each transformation event was similar (as demonstrated by the standard deviation of the results shown in FIG 4).

FIG 5 reports the percent of transformation events expressing GUS in the indicated ranges.

EVALUATION OF ARTIFICIAL MAR IN *ARABIDOPSIS*

A. *Arabidopsis* Transformation Vectors

Act2/GUS/nos (Act2 transcription initiation region/GUS structural gene/nos 3' UTR) constructs were made for testing in a dicot system (*Arabidopsis*). Three vectors were made:

pAct2-bin (SEQ ID NO:30) is a binary vector containing a Act2/GUS/nos cassette, 19S/NPTII/orf25polyA as a selectable marker, and 35S/GFP/nos as an independent reporter gene.

pArAct2Af-bin(SEQ ID NO:31) is identical to pAct2-bin except that it has the MAR dimer of SEQ ID NO:29 positioned 5' to the Act2 transcription initiation region, and the MAR dimer of SEQ ID NO:26 positioned 3' to the nos 3' UTR.

pAfAct2Af-bin (SEQ ID NO:32) is identical to pAct2-bin except that it has the MAR dimer of SEQ ID NO:26 positioned 5'

to the Act2 transcription initiation region the MAR dimer of SEQ ID NO: 33 positioned 3' to the nos 3' UTR.

These vectors enabled testing of two orientations of the artificial MAR dimer in *Arabidopsis*. A schematic of the pArAct2Af-bin and pAfAct2Af-bin constructs is shown in Figure 3.

B. *Arabidopsis* transformation

Arabidopsis transformation was performed according to a protocol provided by Pam Green (van Hoof and Green 1996), which is an adaptation from protocols by Nicole Bechtold (Bechtold *et al.*, 1993), Andrew Bent (Bent *et al.*, 1994) and Takashi Araki (personal communication).

Seeds of ecotype Columbia were planted in 4 inch square pots, covered with window screen mesh, and grown under conditions of 16 hours light/ 8 hours dark at 22°C, fertilizing by subirrigation once a week. The fertilizer consisted of 5 mM KNO_3, 2.5 mM KPO_4 (pH 5.5), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.05 mM Fe•EDTA, 0.07 mM boric acid, 0.014 mM $MnCl_2$, 0.005 mM CuSO4, 0.001 mM $ZnSO_4$, 0.0002 mM $NaMoO_4$, and 0.01 mM NaCl. Plants were thinned to 4 plants per pot and grown until several bolts emerged. When plants were ready to transform, the above soil parts were submerged in infiltration medium (2.2 g/l MS salts, 1X B5 vitamins, 50 g/l sucrose, 2.5 mM MES, pH 5.7, 0.044 M benzylaminopurine, 200 ml/l Silwet L-77™ [Osi Specialties, Inc.] containing *Agrobacterium* cells, placed inside a vacuum desiccator under a vacuum of 400 mm Hg (about 17 inches) for 5 minutes. After quickly releasing the vacuum, pots were drained and placed on their sides in a tray covered with plastic wrap to maintain humidity for 24 hours. The next day the pots were uncovered and set upright. Plants were staked individually and after 2 weeks watering was gradually reduced to allow plants to dry out. Seeds were harvested from each plant individually.

For selection of transformation events, 1-10 mg seeds per plant were surface sterilized by soaking in 10% bleach for 7

minutes while mixing vigorously, followed by three rinses in sterile water, and placed in a flask containing *Arabidopsis* germination medium (MS salts, MS vitamins, 10% sucrose, 2.5 mM 2-[N-morpholino]ethanesulfonic acid [MES], 30 mg/l kanamycin, 50 mg/l vancomycin and 0.1 % Bacto™-Agar [Difco Laboratories, Detroit, MI]). After shaking in continuous light at 90 rpm for 3 days, seeds germinated, and transformants were isolated as green seedlings between 7 and 12 days after germination. Nontransformed seeds produced small bleached seedlings. Transformants were transferred to solid medium (MS salts, B5 vitamins, 10% sucrose, 2.5 mM MES, 15 g/l Phytagar™ [Gibco BRL, Gaithersburg, MD], 30 ml/l kanamycin, 50 mg/l vancomycin) in plates for further selection. After one to two weeks, true transformants were transferred to GA7 vessels (MS salts, B5 vitamins, 0.3 % sucrose, 2.5 mM MES) for one to two weeks prior to planting in soil for production of T1 seed.

C. Southern analyses

Southern analysis was used to identify primary regenerate T2 *Arabidopsis* lines that contained intact copies of the specific gene construct.

Pooled samples of *Arabidopsis* leaf tissue were powdered in liquid nitrogen. The ground tissue was then incubated for three minutes in 500 µl 2X extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 2% 2-mercaptoethanol) at 65°C. Five hundred µl of chloroform/octanol (24:1) was added, the samples were shaken for two minutes, and then spun at 14,000 ´ g in a microcentrifuge and the supernatant was removed. The chloroform/octanol extraction was repeated. One ml of precipitation buffer (1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% 2-mercaptoethanol) was added to the supernatant and then incubated at room temperature for 60 minutes. The DNA was pelleted by centrifugation at 3500g for 5 minutes in a microcentrifuge. The pellet was drained and resuspended in 200 µl 1.0 M NH_4OAc. One hundred µl 7.5 M NH_4OAc and 1 ml

isopropanol were added, the samples incubated on ice for 5 minutes and then centrifuged at 14,000 ´ g for 5 minutes. The pellet was drained and resuspended in 200 µl TE. 100 µl 7.5 M NH_4OAc and 1 ml isopropanol were added and incubated on ice for 5 minutes then centrifuged at 14,000 ´ g for 5 minutes. The pellet was drained and rinsed with 70% ethanol and dried in a Speed Vac (Savant Instruments Inc., Farmingdale, NY). The dried pellet was resuspended in 20 µl TE (10 mM TRIS, 1 mM EDTA, pH 8.0).

Southern analysis was conducted on 29 ArAct2Af T2 lines and 24 AfAct2Af T2 lines. One microgram of DNA from the ArAct2Af plants was digested with the restriction enzyme *Xba*I using conditions suggested by the manufacturer (Bethesda Research Laboratory, Gaithersburg, MD) and separated by agarose gel electrophoresis, which should result in a 4.6 kb hybridization product, if the gene construct is intact, when radiolabeled with a probe specific for the GUS coding region. Similar to the ArGOS2Af rice plants, the 4.6 kb fragment should consist of the artificial MAR in the reverse orientation, the Act2 promoter, the GUS coding region, the nos 3′ UTR and the artificial MAR in the forward orientation. The DNA was blotted onto nylon membranes as described by Southern (1975, 1989). Radiolabeled probe DNA was hybridized to the genomic DNA on the blots using 50 ml of minimal hybridization buffer (10% polyethylene glycol, 7% sodium dodecyl sulfate, 0.6x SSC, 10 mM sodium phosphate, 5 mM EDTA and 100 mg/ml denatured salmon sperm DNA) was heated to 60°C and mixed with the denatured radiolabeled probed prior to being added to the blots for overnight hybridization at 60°C. The blots were washed at 60°C in 0.25X SSC and 0.2% SDS for 45 minutes, blotted dry and exposed to XAR-5 film with two intensifying screens overnight.

The expected 4.6 kb hybridization product was detected in twenty-six of the twenty-nine ArAct2Af lines. A second Southern blot was generated to determine the copy number of the ArAct2Af construct. The ArAct2Af DNA was digested with

the restriction enzymes *Sst*I and *Xho*I which cut the construct near the right and left borders of the T DNA. The blots were radiolabeled with probes specific for the artificial MAR and the DNA from the left border to the *Xho*I site 800 bp downstream. A single copy of the ArAct2Af construct will have three hybridization products: two fragments of unknown size, consisting of the left and right border DNA, and the 8.9 kb fragment which is the DNA internal to the borders. Twenty-two of the twenty-nine lines had three or fewer hybridization products, indicating that a single copy of the ArAct2Af construct was present.

The DNA from the AfAct2Af plants was digested with the restriction enzyme *Xba*I which, if the construct remains intact, should result in a 5.7 kb hybridization product when radiolabeled with a probe specific for the GUS coding region. The 5.7 kb fragment should consist of the artificial MAR in the forward orientation, the Act2 promoter, the GUS coding region, the nos 3' UTR and the artificial MAR in the forward orientation. It also includes the green fluorescent protein coding region and the nos 3' UTR. The expected 5.7 kb hybridization product was detected in all of the twenty-four AfAct2Af lines.

A second Southern blot was generated to determine the copy number of the AfAct2Af construct. The AfAct2Af DNA was digested with the restriction enzymes *Sst*I and *Xho*I which cut the construct near the right and left borders of the T DNA. The blots were radiolabeled with probes specific for the artificial MAR and the DNA from the left border to the *Xho*I site 800 bp downstream. A single copy of the AfAct2Af construct will have three hybridization products: two fragments of unknown size, consisting of the left and right border DNA and the 8.9 kb fragment which is the DNA internal to the borders. Fifteen of the twenty-four lines had three or fewer hybridization products indicating that a single copy of the AfAct2Af construct was present.

The non-Mar control lines, Actbin, were also analyzed by Southern analysis. The DNA from thirty-four Actbin T2 lines was digested with the restriction enzyme PstI which should, if the construct remains intact, result in a 3.4 kb hybridization product when radiolabeled with a probe specific for the GUS coding region. The 3.4 kb fragment should consist of the Act2 promoter, the GUS coding region, and the nos 3' UTR. The expected 3.4 kb hybridization product was detected in thirty of the thirty-four Actbin lines. A second Southern blot was generated to determine the copy number of the Actbin construct. The DNA from the Actbin lines was digested with the restriction enzymes *Sst*I and *Xho*I which cut the construct near the right and left borders of the T DNA. The blots were radiolabeled with probes specific for the nos 3'UTR and the DNA from the left border to the *Xho*I site 800 bp downstream. A single copy of the Actbin construct will have three hybridization products: two fragments of unknown size, consisting of the left and right border DNA and the 8.2 kb fragment which is the DNA internal to the borders. Nine of the thirty-four lines had three or fewer hybridization products indicating that a single copy of the Actbin construct was present.

D. GUS analysis

For growing *Arabidopsis*, T2 seed was germinated *in vitro* on MS medium containing 90 mg/l kanamycin and 3 week old kanamycin resistant seedlings were harvested. Two batches of 30 seedlings per transformation event were used for GUS analysis and additional seedlings were used to extract DNA.

For analyses of GUS activity, leaf samples were powdered in liquid nitrogen and samples of approximately 400 mg of tissue were placed in microfuge tubes. Two independent samples from each leaf sample was processed. The tissue was either stored at -70°C or extracted immediately. GUS was extracted by mixing the powdered tissue with GUS lysis buffer (Jefferson *et al.*, 1987) modified by the addition of 1%

polyvinylpolypyrrolidone (hydrated in the buffer for at least one hour) and 20% glycerol. After incubation on ice for at least 10 min, the samples were centrifuged at 16,000 ´ g for 10 min. The supernatants were recovered and centrifuged a second time as described above. The supernatants were recovered and frozen on dry ice and stored at -70°C. Experiments showed that GUS activity was stable for at least 4 freeze-thaw cycles when stored in the buffer described above (W.M. Ainley, unpublished). GUS activity was measured using a GUS-Light™ kit (Tropix, Inc., Bedford, MA). Five µl samples of undiluted extract or of extract diluted so that the luminescence was within the range measured by the luminometer was added to 195 µl of the GUS-Light™ Reaction Buffer. Luminescence was integrated for 5 sec after a 5 sec delay. Protein was measured with the assay developed by Bradford (1976) using human serum albumin as the standard. GUS activity was normalized between experiments using a GUS standard obtained from SIGMA. The amount of plant protein in the standards and plant samples was the same; protein was adjusted where necessary using extracts of nontransformed plants.

FIGS 6 and 7 report the results the GUS analysis. FIG 6 reports expression observed for independent transformation events expressing either the base, non-MAR construct (Actbin) or the base construct flanked by the artificial MAR in the indicated orientation (ArAct2Af or Af-Act-Af). Standard deviations indicate the variance between plants harvested from different plates. Arrows indicate the relative orientations of the MARs.

E.

In FIG 7, percent of transformation events expressing GUS in the indicated ranges is shown.

E. Characterization of transgenic plants expressing the reporter gene constructs

T2 *Arabidopsis* plants were analyzed. The plants were evaluated for segregation of kanamycin resistance to determine

insert copy number. A few events did not fall into either one or two insert categories as determined by chi square analyses. Of the remaining plants, 72% had a single insert. Most of the inserts had multiple copies of the transgene. Although the *Arabidopsis* transformation events were grown under controlled environments, there were some occasional differences in the relative growth of plants between the duplicate plates. In those cases, the events were either not used or were grown again. The coefficient of variance of the expression determined from duplicate plates range of between 1 and 46%, with 89 percent below 20 percent (Figure 6).

Generally, all *Arabidopsis* transformation events tested for a construct were grown and analyzed together. Although the growth of the plants was under controlled environments, the possibility remained that the differences observed between transformation events expressing the constructs was due to environmental differences occurring between different experiments. To eliminate this possibility, three of the highest expressing events from the three sets of *Arabidopsis* transformation events were grown together and reanalyzed. This analyses confirmed the earlier differences between the sets of transformation events.

SUMMARY OF EFFECTS OF THE ARTIFICIAL MAR ON TRANSGENE EXPRESSION

In both rice and *Arabidopsis*, the average expression level of transformation events expressing the MAR-containing constructs expressed at a higher level than those lacking the MAR elements (Table 3). In *Arabidopsis*, orientation of the MARs tested influenced the level of expression. Plants containing constructs in which the MARs were in the same orientation on either side of the GUS gene construct expressed GUS at higher levels than plants containing constructs in which the MARs were oriented in opposite orientations.

There appeared to be a proportionally higher expression level in the higher expressors than in the lower expressors in

each set of transformation events. To document this, expression of the upper quartile of the expressors was compared (Table 3). Based on this data, the upper quartile of the transformation events expressing the AfAct2Af construct produce GUS protein at levels 5.6 times higher than in the upper quartile of events expressing the Actbin construct. The artificial MARs in opposite orientations in both rice and *Arabidopsis* enhance expression approximately two-fold over the respective constructs lacking MARs.

Table 3

Comparison of the expression of transformation events expressing either the base constructs or base constructs flanked by the artificial MARs.

	CONSTRUCT				
	GOS2	ArGOS2Af	ActBin	Ar-Act-Af	Af-Act-Af
species transformed	rice	rice	*Arabidopsis*	*Arabidopsis*	*Arabidopsis*
method of transformation	particle bombardment	particle bombardment	*Agrobacterium*	*Agrobacterium*	*Agrobacterium*
number of transformation events analyzed	28	25	28	21	22
average	357	713	2,313	3,673	9,042
median	95	317	2,205	2,772	5,730
upper quartile range	258-3,076	1,674-2,661	3,047-5,311	6,240-10,614	15,595-27,179
upper quartile average	1,169	2,099	4,056	7,812	22,688
relative average expression	1.0	2.0	1.0	1.6	4.0
relative upper average quartile expression	1.0	1.8	1.0	1.9	5.6

averages for the upper quartile of transformation event expression levels were statistically different (p is less than or equal to 0.05) based on t-test analyses. (Gopal K. Kanji 1995)

Previous published studies (reviewed by Holmes-Davis and Comai, 1998) have shown that, with the exception of one MAR, all MARs tested to date enhance expression of reporter genes. This study represents the first report that a MAR constructed using elements found preferentially in MARs can enhance expression in plant species representing both monocotyledonous and dicotyledonous plants.

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Claims

1. An isolated DNA molecule comprising bp 11 to 309 of SEQ ID NO: 1.

2. A DNA construct comprising, in the 5' to 3' direction: a transcription initiation region functional in plant cells, a structural gene operatively associated with the transcription initiation region, a 3' untranslated region, and a matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 positioned either 5' to said transcription initiation region or 3' to said structural gene.

3. A DNA construct of claim 2 wherein said matrix attachment region comprises two or more tandem copies of bp 11 to 309 of SEQ ID NO:1.

4. A DNA construct of claim 2 wherein a first matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 is positioned 5' to said transcription initiation region and a second matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 is positioned 3' to said structural gene.

5. A DNA construct of claim 4 wherein each of said matrix attachment regions is comprised of two or more tandem copies of bp 11 to 309 of SEQ ID NO:1.

6. A method of making recombinant plant cells having increased expression of structural genes introduced therein which comprises:
transforming a plant cell capable of regeneration with a DNA construct of claim 2.

7. A method of claim 6 wherein the matrix attachment region in said DNA construct comprises two or more tandem copies of bp 11 to 309 of SEQ ID NO:1.

8. A method of claim 6 wherein the DNA construct includes a first matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 positioned 5' to said transcription initiation region and a second matrix attachment region comprised of bp 11 to 309 of SEQ ID NO: 1 positioned 3' to said structural gene.

9. A method of claim 8 wherein said first and second matrix attachment regions are each comprised of two or more tandem copies of bp 11 to 309 of SEQ ID NO:1.

10. A transformed plant cell containing DNA of bp 11 to 309 of SEQ ID NO:1.

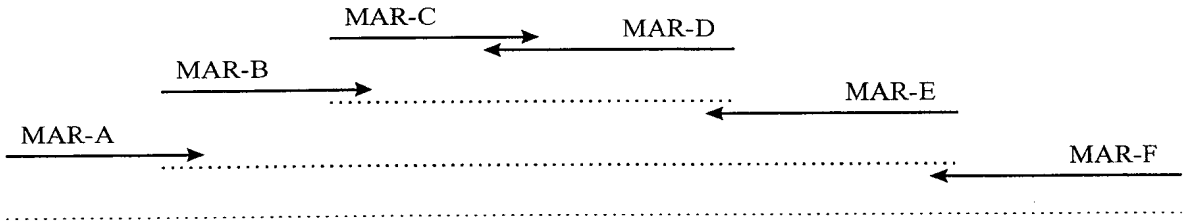
MAR-C
MAR-D
MAR-B
MAR-E
MAR-A
MAR-F

FIG 1

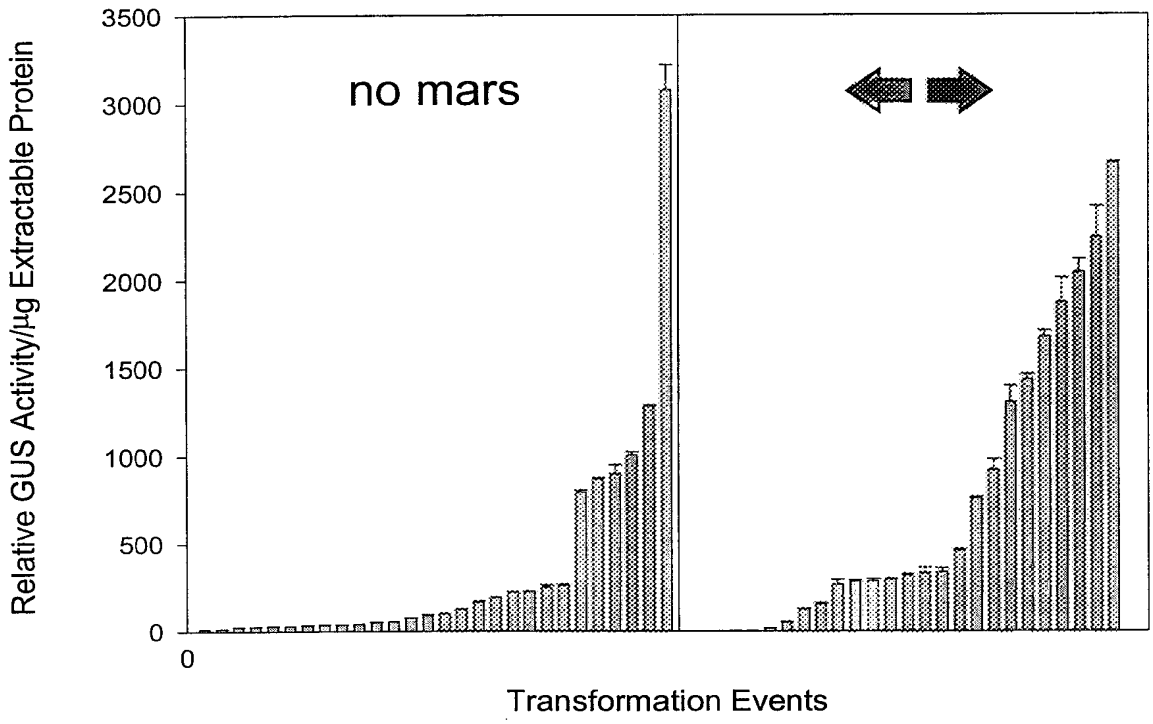
3500
3000
2500
2000
1500
1000
500
0
0
no mars
Relative GUS Activity/µg Extractable Protein
Transformation Events

FIG 3

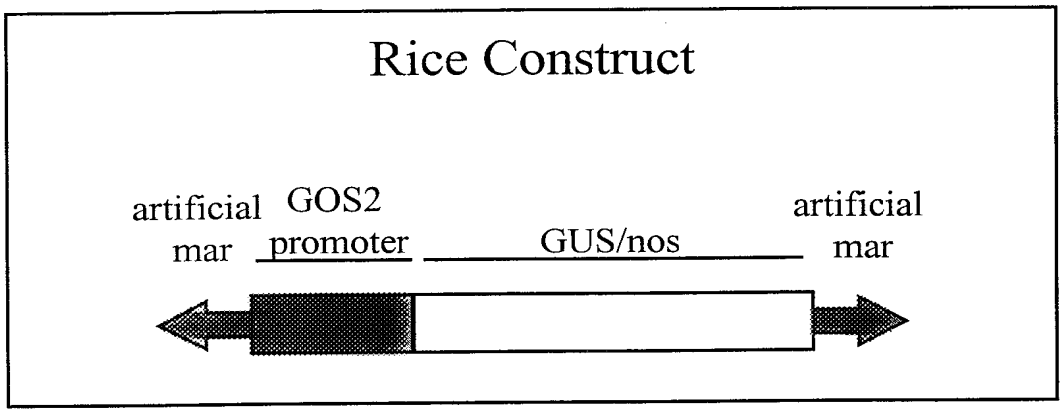
Rice Construct
artificial mar
GOS2 promoter
GUS/nos
artificial mar

FIG 2

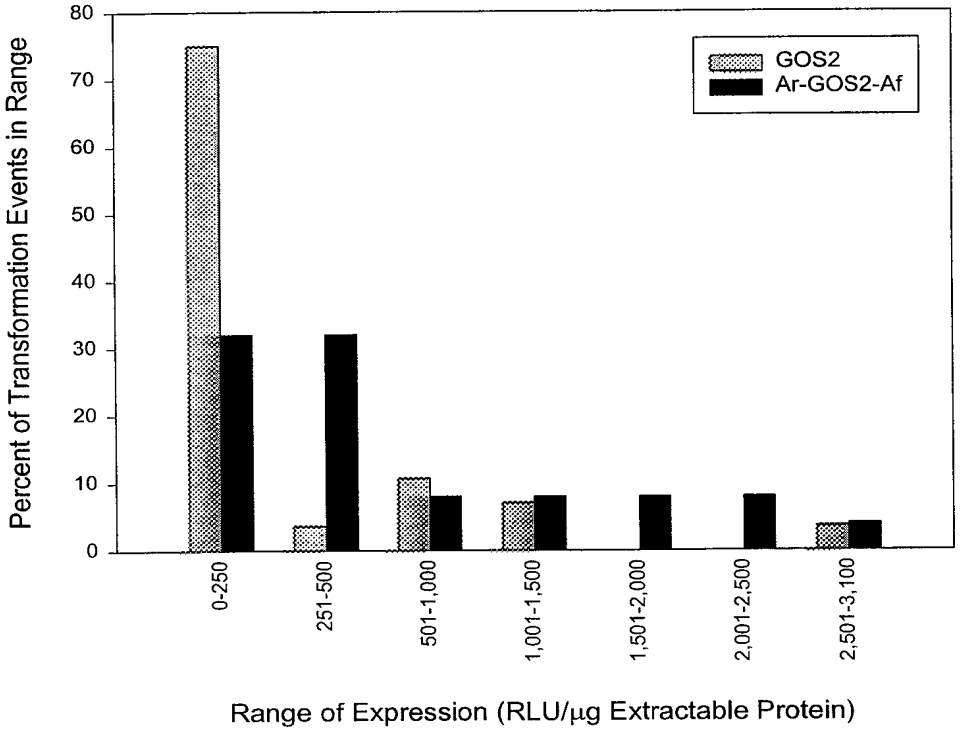
Percent of Transformation Events in Range
80
70
60
50
40
30
20
10
0
GOS2
Ar-GOS2-Af
0-250
251-500
501-1,000
1,001-1,500
1,501-2,000
2,001-2,500
2,501-3,100
Range of Expression (RLU/µg Extractable Protein)

FIG 4

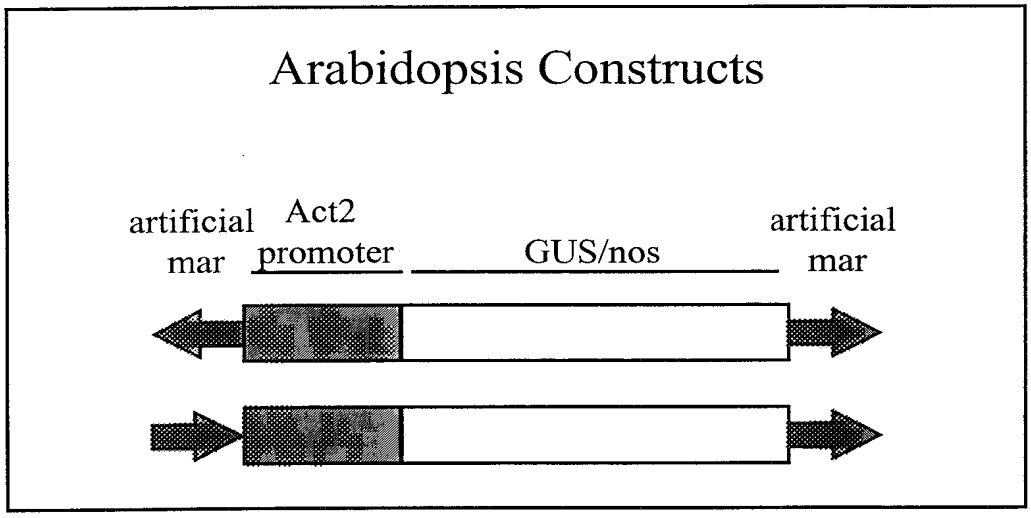
Arabidopsis Constructs
artificial mar
Act2 promoter
GUS/nos
artificial mar

FIG 5

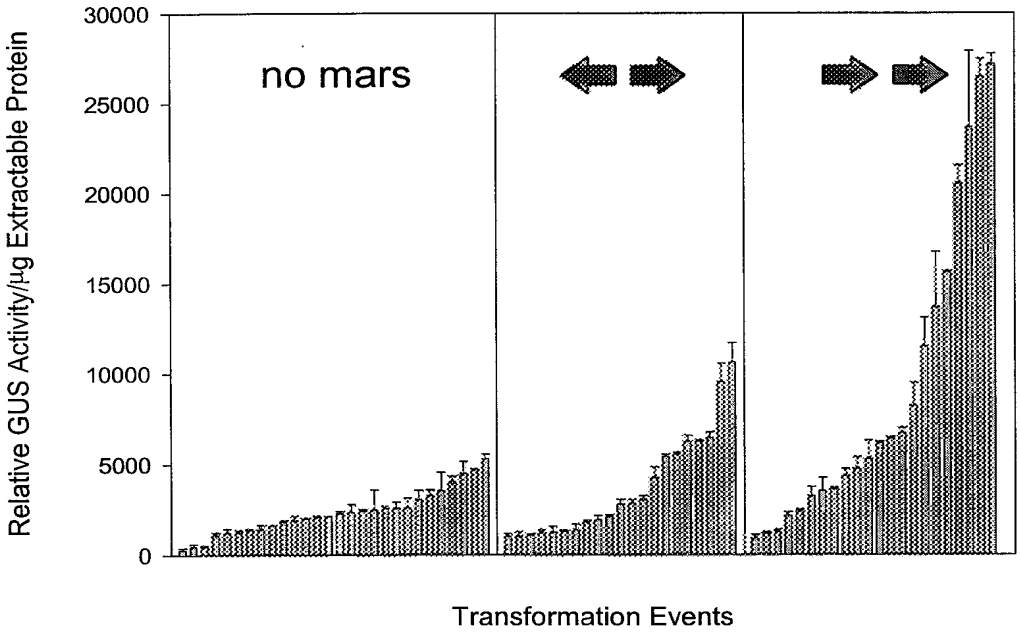
Relative GUS Activity/µg Extractable Protein
30000
25000
20000
15000
10000
5000
0
no mars
Transformation Events

FIG 6

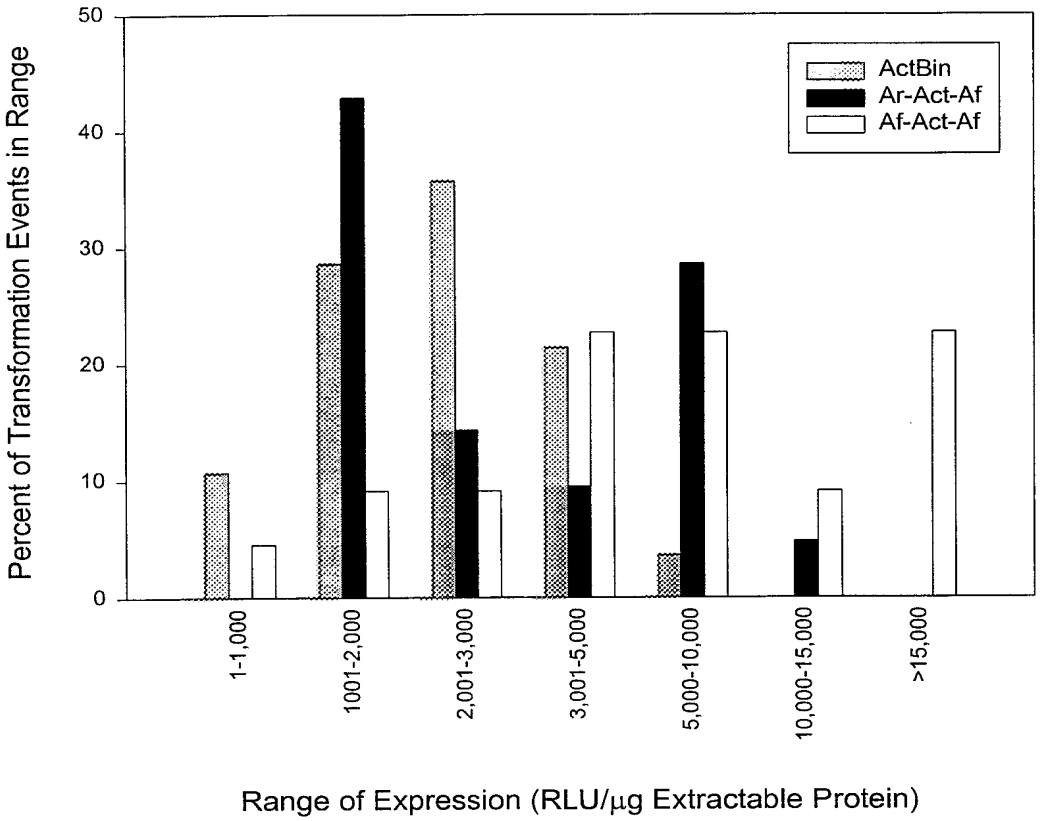
50
40
30
20
10
0
Percent of Transformation Events in Range
ActBin
Ar-Act-Af
Af-Act-Af
1-1,000
1001-2,000
2,001-3,000
3,001-5,000
5,000-10,000
10,000-15,000
>15,000
Range of Expression (RLU/μg Extractable Protein)

FIG 7

SEQUENCE LISTING

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Ainley, W. Michael
Cowen, Neil W.
Welter, Mary E.
Woosley, Aaron T

<120> ARTIFICIAL MATRIX ATTACHMENT REGION FOR INCREASING EXPRESSION OF GENES INTRODUCED IN PLANT CELLS

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/28123

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/29 C12N15/67 C12N15/63 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIU J -W ET AL: "THE INFLUENCE OF TWO PLANT NUCLEAR MATRIX ATTACHMENT REGIONS (MARS) ON GENE EXPRESSION IN TRANSGENIC PLANTS" MOLECULAR AND GENERAL GENETICS,DE,SPRINGER VERLAG, BERLIN, vol. 39, no. 1, 1 January 1998 (1998-01-01), pages 115-123, XP002910494 ISSN: 0026-8925 the whole document --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2000	10/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ALCONADA RODRIG.., A

3

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3

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 99/28123

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	SANDER M ET AL: "Drosophila topoisomerase II double-strand DNA cleavage: analysis of DNA sequence homology at the cleavage site." NUCLEIC ACIDS RESEARCH, (1985 FEB 25) 13 (4) 1057-72. , XP000891514 cited in the application	1-10

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